FURTHER STUDIES OF ACTIVATION-INACTIVATION COUPLING IN *MYXICOLA* AXONS

INSENSITIVITY TO CHANGES IN CALCIUM CONCENTRATION

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ABSTRACT In *Myxicola* axons subjected to moderate depolarizations the sodium inactivation time constants obtained from the decay of sodium current during a maintained depolarization (τ_h^d) are substantially smaller than inactivation time constants determined at the same potential from the effect of changes in the duration of conditioning prepulses (τ_h^p). This report extends these observations to positive membrane potentials and demonstrates that for sufficiently large depolarizations τ_h^d and τ_h^p become comparable. The ratio of inactivation time constants, τ_h^p/τ_h^d, is unaffected by changes in $[Ca^{++}]$ provided total divalent cation concentration is maintained constant, while changes in total divalent ion concentrations produce simple voltage shifts comparable to those obtained from measurement of membrane sodium or potassium conductances. Sodium inactivation delay was quantitatively determined as a function of membrane potential, and found to be similarly unaffected by changes in $[Ca^{++}]$ at constant total divalent ion concentration. Inactivation delay is, however, directly proportional to the activation rate constant over a wide range of potentials.

INTRODUCTION

A number of theoretical models of the behavior of the sodium channel have ascribed a specific critical role to calcium ions in the gating process (Goldman, 1964; Offner, 1970; Fishman et al., 1971; Moore and Jakobsson, 1971). In *Myxicola* axons, however, equimolar substitution of Mg^{++} for Ca^{++} has no detectible effect on voltage-dependent membrane parameters (Schauf, 1975). Except for a lower sensitivity of steady-state sodium inactivation, the result of changing external $[Ca^{++}]$ is consistent with a simple screening hypothesis in which no detectible Ca^{++} binding occurs and the surface charge density is -0.013 charges $/Å^2$ (Schauf, 1975). However, these data were obtained solely from experimental procedures in which there is no serious deviation from Hodgkin-Huxley kinetics.

Voltage-clamp studies of *Myxicola* axons (Goldman and Schauf, 1973; Schauf, 1973), lobster axons (Oxford and Pooler, 1975), and frog nerve (Peganov, et al., 1974) have demonstrated that sodium inactivation time constants measured from the decay of sodium current during a maintained depolarization are substantially smaller than

the inactivation time constants determined at the same membrane potential using conditioning prepulses. Such data is difficult to reconcile with the existence of independent gating processes for sodium activation and inactivation. With sufficiently brief prepulses, clear deviations from simple exponential behavior can be observed both during inactivation (Armstrong, 1970; Goldman and Schauf, 1972) and on reactivation (Schauf, 1974). A formal quantitative description of *Myxicola* kinetics in terms of a generalized second order variable has been presented (Goldman, 1975). Further support for the concept that activation and inactivation are not independent processes has been recently obtained from studies of gating currents in squid axons (Armstrong and Bezanilla, 1974; Bezanilla and Armstrong, 1975) and from the effects of pancuronium (Yeh and Narahashi, 1975).

A complete analysis of the response of *Myxicola* axons to Ca^{++} requires investigation of all features of sodium channel gating, including those aspects anomalous relative to the Hodgkin-Huxley kinetics. Perhaps here an intimate involvement of Ca^{++} with the gating processes might be reflected in some Ca^{++} sensitivity inconsistent with simple screening of membrane surface charge.

METHODS

Myxicola giant axons were voltage-clamped by methods previously described (Binstock and Goldman, 1969). Compensated feedback was used in all experiments. The reference artificial sea water (ASW) solution had the composition: 430 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM tris (hydroxymethyl) aminomethane. The pH was adjusted by the addition of sufficient 6M HCl prior to each experiment to be 7.8 ± 0.05 at a temperature of 5.0 ± 0.5°C. The experiments requiring changes in $[Ca^{++}]$ were generally performed at constant total divalent ion concentrations (60 mM) by reciprocal alteration of $[Mg^{++}]$. General procedures were similar to those reported elsewhere (Schauf, 1975). Currents were recorded first in the reference ASW, then in a test solution with altered $[Ca^{++}]$, and finally in the presence of 10^{-6}M tetrodotoxin and appropriate amounts of Ca^{++} using a pulse protocol identical to that used initially. Sodium currents were obtained by the subtraction of corresponding records. Measurements of $\tau_h^i(V)$ and $\tau_h^s(V)$ were made on an individual axon in reference ASW and in the test solutions so that the axon served as its own control. In a few cases experiments were performed in Mg^{++} free solutions with varied $[Ca^{++}]$.

RESULTS AND DISCUSSION

Prior to investigating the effects of changes in $[Ca^{++}]$, we wished to extend as far as possible the voltage range over which inactivation time constants in *Myxicola* have been determined both during step depolarizations (τ_h^i) and from the effect of prepulses (τ_h^s). The major reason for this effort was to see whether τ_h^i and τ_h^s would become equal at large depolarizations.

Our determination of the voltage dependence of τ_h^i was identical to that previously described (Goldman and Schauf, 1973). Nevertheless, its measurement was essential so that comparison data could be available on the same axons. Measurement of $\tau_h^s(V)$ involves using a prepulse of variable duration, followed by a fixed test pulse (V_t).

The amplitude of the peak sodium current during V_t is monitored as prepulse duration is increased. For hyperpolarizing prepulses or moderate depolarizations there is no difficulty in fitting an exponential function to the changing sodium current, because the magnitude of the inactivation delay (Armstrong, 1970; Goldman and Schauf, 1972) is negligible compared to the overall time course of inactivation. The initial deviation is simply neglected in the determination of τ_h^p.

However for prepulses more depolarized than those used by Goldman and Schauf (1972), this procedure is no longer completely satisfactory. At prepulse potentials more positive than 0 mV the overall rate of inactivation becomes sufficiently rapid that the presence of the initial delay (which is only decreasing slowly with increasing potential in this range) prevents us from obtaining an adequate fit. A much better description of the data is provided if one arbitrarily assumes the existence of a finite dead time and uses the relation.

$$\frac{G_{Na}}{G_{Na}^0} = \begin{cases} 1 & t \leq t_d \\ \exp[-(t - t_d)/\tau_h^p] & t > t_d \end{cases} \quad (1)$$

This relation was employed in a curve-fitting program to analyze all experimental data, not just that at positive prepulse potentials. Fig. 1 shows that the measurements are essentially superimposable with the data reported previously by Goldman and

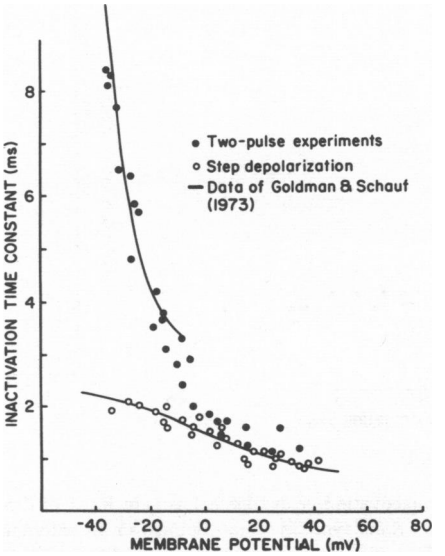

FIGURE 1 Inactivation time constants measured from the effect of prepulse duration on the peak sodium current during a subsequent fixed test pulse (τ_h^p, solid symbols), and from curve fitting the decline in sodium current during a step depolarization (τ_h^s, open symbols), plotted as a function of membrane potential. Values of τ_h^p were obtained by assuming the existence of a finite dead time. The solid lines are calculated using Eqs. 12–14 of Goldman and Schauf (1973) for the region over which they obtained data. Data from four experiments using 10 mM Ca^{++} and 50 mM Mg^{++} in the external solution.

Schauf (1973) over most of the potential range. However, at potentials near zero, and most noticeably at positive potentials, the inactivation time constants, τ_h^s and τ_h^p, begin to become comparable to one another.

The inequality of τ_h^p and τ_h^s between −40 mV and approximately 0 mV is difficult to explain unless there is more than one pathway to the inactive state. The present extension of these observations suggests that only a single pathway may be primarily utilized at positive potentials.

In addition to demonstrating an equality of τ_h^s and τ_h^p for large positive prepulses, the procedure of Eq. 1 enables a quantitative determination of inactivation delay, defined simply as t_d. The results are presented in Fig. 2 (solid symbols). Although the scatter is large, the inactivation delay seems to be a constant multiple (1.6) of the activation time constant expression derived earlier by Goldman and Schauf (1973). Such a quantitative correlation between inactivation delay, and the rate of activation seems in itself strong evidence for coupling between the activation and conditioning processes.

The effects of alterations in calcium concentration on these determinations were examined in six experiments in which there was a reciprocal alteration of $[Ca^{++}]$ and $[Mg^{++}]$ so as to maintain total divalent cation concentration constant and thus eliminate the effect of changes in surface potential (Schauf, 1975).

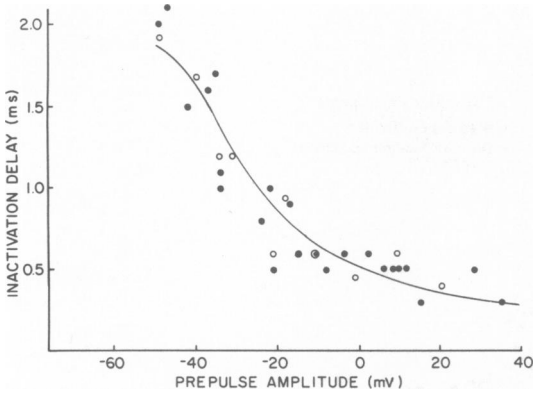

FIGURE 2

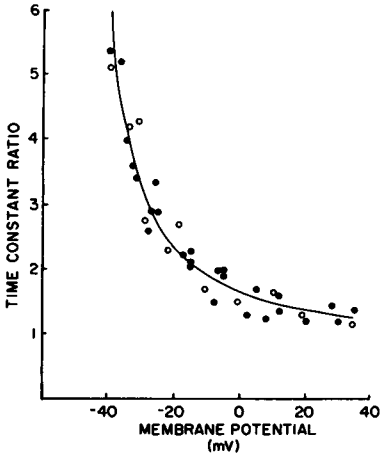

FIGURE 3

FIGURE 2 Value of the inactivation delay, defined as t_d in Eq. 1 of the text, plotted as a function of prepulse potential. Solid symbols were obtained in an external solution containing 10 mM Ca^{++}, 50 mM Mg^{++}; open symbols either in a 60 mM Mg^{++}, Ca^{++} free solution or in a 60 mM Ca^{++}, Mg^{++} free solution. The solid line is 1.6 τ_m where τ_m is defined by Eqs. 9 and 10 of Goldman and Schauf (1973) and the relation $\tau_m = (\alpha_m + \beta_m)^{-1}$.

FIGURE 3 Values of the time constant ratio τ_h^p/τ_h^s plotted as a function of membrane potential. Solid symbols were obtained in 10 mM Ca^{++}, 50 mM Mg^{++}; open symbols in either 0 mM Ca^{++}, 60 mM Mg^{++} or 60 mM Ca^{++}, 0 mM Mg^{++}. The solid line was computed from Eqs. 12–14 of Goldman and Schauf (1973) over the range −40 mV to −15 mV and extended by eye to fit the remaining data for 60 mM divalent ions.

Figs. 2 and 3 show the results of these experiments. In Fig. 2, referred to previously the open symbols are inactivation delays measured in 0 mM Ca^{++} 60 mM Mg^{++}, or 60 mM Ca^{++} 0 mM Mg^{++} solutions. There is no difference from the remainder of the data. In Fig. 3 the time constant ratio τ_h^p/τ_h^i is plotted for a 10 mM Ca^{++}, 50 mM Mg^{++} solution (solid symbols) and under conditions of altered [Ca^{++}] but constant total divalent cation concentration (open symbols). With constant divalent cation concentration the ratios are not affected by changes in [Ca^{++}].

A number of experiments were also performed in which [Ca^{++}] was altered in Mg^{++} free solutions. The inactivation time constant ratios were simply translated along the voltage axis by amounts comparable to those obtained from the shift in the conductance-voltage curves in agreement with the screening of membrane surface charge previously reported (Schauf, 1975).

These experiments have failed to demonstrate any specific interaction of Ca^{++} with those aspects of *Myxicola* behavior inconsistent with Hodgkin-Huxley kinetics. Thus, the only observation in this preparation which remains at variance with a simple screening of membrane surface charge is the lower magnitude of the h_∞ shift compared to other parameters (Schauf, 1975).

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